



## DNA barcoding assessment of genetic variation in two widespread skinks from Madagascar, *Trachylepis elegans* and *T. gravenhorstii* (Squamata: Scincidae)

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### Abstract

*Trachylepis elegans* and *T. gravenhorstii* are two of the most widespread reptiles in Madagascar, inhabiting a wide variety of habitats. Previous studies have indicated a considerable mitochondrial DNA (mtDNA) variation within these species, but the geographic distribution of the major haplotype lineages is poorly known. Herein we analyse the phylogeography of these lizards based on 107 sequences of the mitochondrial cytochrome oxidase subunit I gene, 101 of which newly determined. As in previous mtDNA assessments, *T. elegans* and *T. gravenhorstii* were not reciprocally monophyletic, although recent analyses including nuclear markers indicated their probable monophyly, respectively. The main lineages within *T. gravenhorstii* were found in strict allopatry and could be divided into a subclade of roughly northern and eastern distribution (lineages 1 and 2) and a subclade of roughly southern and western distribution (lineages 3, 4a, 4b, and 5, plus *T. elegans*). Our data serve to identify more precisely the probable contact zones among *T. gravenhorstii* lineages. The two main mtDNA clades (represented by lineages 2 and 3, respectively) can be expected to come into close contact in the area of the upper Mangoro river and Alaotra Lake, and (lineages 2 and 4a) in the Southern Central East between Mananjary and Ranomafana. Future studies intensively sampling these contact zones have the potential to assess hybridization and admixture among these lineages, and to test whether they are deep conspecific lineages of *T. gravenhorstii* as currently understood, or might represent distinct species.

**Key words:** Squamata, Scincidae, Lygosominae, *Trachylepis*, Madagascar, cytochrome oxidase subunit I, biogeography

### Introduction

The “*Mabuya* clade” represents a species rich pantropical radiation of mostly medium sized lizards of the family Scincidae, characterized by a highly conserved morphology combined with a relatively convoluted taxonomic history. Phylogenetic studies published during the last decade led to splitting the former genus *Mabuya* sensu lato into four geographically distinct monophyletic genera, i.e., *Eutropis* in Asia, *Trachylepis* mainly in Africa and Madagascar, *Mabuya* sensu stricto in the Neotropics and *Chioninia* in the Cape Verde archipelago (Mausfeld *et al.* 2002, Carranza & Arnold 2003), and a phylogenetic meta-analysis of squamates revealed two additional genera (*Dasia* in Asia and *Eumecia* in Southern Africa) nested within the “*Mabuya* clade” (Pyron *et al.* 2013).

Recent molecular studies focusing on these different genera have shown that most of the taxa alleged to be common species with wide geographic distributions were actually constituted by complexes of several cryptic or pseudo-cryptic species with relatively restricted allopatric distributions (Datta-Roy *et al.* 2012; Hedges & Conn 2012; Miralles *et al.* 2009, 2011, Miralles & Carranza 2010; Sindaco *et al.* 2012). These observations led us to investigate herein the molecular differentiation within two Madagascar-endemic species of *Trachylepis* that are among the most widespread and common Malagasy reptiles, namely, *T. elegans* (Peters) and *T. gravenhorstii* (Duméril & Bibron).

With the exception of dense and shady rainforest, these two species populate a large range of habitats. *Trachylepis elegans* occurs in lowland areas especially in the west of the island, while *T. gravenhorstii* in addition to the former habitats also occurs in numerous highland areas up to at least 1400 m above sea level. These two species are also common in disturbed and anthropogenic habitats and are often found even within densely populated cities such as Madagascar's capital, Antananarivo. Together with a third species, *T. madagascariensis*, they are characterized by a trapezoidal shaped subocular scale and compose the phenetic *T. elegans* species group (Brygoo 1983; Glaw & Vences 2007) which forms the sister clade to the remaining endemic *Trachylepis* species of Madagascar (Lima *et al.* 2013).

Since the revision of Brygoo (1983), the taxonomy of these morphologically quite similar lizards has not been thoroughly revised. Given the high molecular variation found especially within *T. gravenhorstii* (Boumans *et al.* 2007) it might be suspected that they represent species complexes, as has been observed with other widespread Malagasy reptiles thought to occur over different ecoregions in Madagascar (e.g., Raxworthy & Nussbaum 2006; Raxworthy *et al.* 2007; Rocha *et al.* 2010; Florio *et al.* 2012; Gehring *et al.* 2012a, 2012b).

Morphologically, *T. elegans* and *T. gravenhorstii* are superficially similar but distinguished by significant differences in various morphometric, meristic and chromatic characters, such as longer limbs, longer head, fewer dorsal and ventral scales, fewer scales around midbody, more scales under toes, lower number of infralabials, split vs. fused frontoparietal, and reddish markings on the neck in *T. elegans* (Brygoo 1983; Andreone & Greer 2002; Lima *et al.* in press). These morphological differences are also maintained in areas of sympatry. While mitochondrial data (Boumans *et al.* 2007) resulted in a non-monophyletic arrangement of the two species, a multi-gene analysis including nuclear genes weakly supported their reciprocal monophyly (Lima *et al.* 2013). Combined, these morphological and genetic analyses, confirm their status as separate species.

Herein we extend the analyses of Boumans *et al.* (2007) who sequenced 16 specimens of *T. elegans* and 30 specimens of *T. gravenhorstii* for the mitochondrial 16S rRNA gene. We assembled a new data set of a fragment of the mitochondrial cytochrome oxidase subunit I (COI) gene which corresponds to the standard DNA barcode marker (Hebert *et al.* 2003), based on sequences of 23 *T. elegans* and 84 *T. gravenhorstii* covering most of the range of these two species. Our goal is not to resolve the taxonomy of these lizards but to locate more precisely the areas where the ranges of the different mtDNA lineages abut, and identify more precisely the cases of sympatric co-occurrence, and thereby provide the basis for more in-depth future sampling in these contact zones.

## Material and methods

Tissue samples were collected in the field, either by excising muscle tissue from the thigh of freshly killed specimens, or by sampling autotomized tail tips and subsequently releasing the individuals. Samples are here labelled with the field number of the respective voucher specimen or tissue sample, using the following acronyms: FGZC, FGMV, ZCMV: field numbers of Frank Glaw and Miguel Vences (voucher specimens largely deposited in the collections of the Zoologische Staatssammlung München [ZSM] in Germany, or in the Département de Biologie Animale of the Université d'Antananarivo [UADBA] in Madagascar). Other acronyms used are: MVTIS, tissue sample in the collection of M. Vences; DRV, field number of David R. Vieites; PSG, sample number of Sebastian Gehring. Geographic regions within Madagascar are named after Boumans *et al.* (2007) and Glaw & Vences (2007).

Tissue samples were preserved in 95–99% ethanol, and total genomic DNA was extracted from the tissue samples using proteinase K digestion (10 mg/ml concentration) followed by a standard salt-extraction protocol (Bruford *et al.* 1992). We used the reptile-specific primers RepCOI-F (TNT TMT CAA CNA ACC ACA AAG A) and RepCOI-R (ACT TCT GGR TGG CCA AAR AAT CA) of Nagy *et al.* (2012) to amplify a COI fragment of 612 bp. The thermocycling profile comprised an initial denaturation at 94°C for 2:20 min, followed by 35 cycles of denaturation (94°C for 30 s), annealing (45–49°C for 45 s), elongation (72°C for 90 s), and a final elongation step at 72°C for 10 min. Reactions were performed in a final volume of 12.5 µl using the following concentration of reagents: 0.24 µM of each primer, 200 µM of dNTP, 1xPCR buffer, and 0.4 units of GoTaq DNA polymerase (Promega, Mannheim, Germany). PCR products were cleaned with enzymatic purification: 0.15 units of Shrimp Alkaline Phosphatase (SAP) and 1 unit of Exonuclease I (New England Biolabs, Frankfurt am Main, Germany) incubated for 15 min at 37°C followed by 15 min at 80°C. Purified PCR products were sequenced on an automated

DNA sequencer (Applied Biosystems ABI 3130XL). Sequencing reactions (10 µl) contained 0.2 or 0.3 µl of PCR product, 0.5 µl of BigDye 3.1 (Applied Biosystems, Darmstadt, Germany), and 0.3 µM of primers. Sequences were checked for errors using CodonCode Aligner (CodonCode Corporation, Dedham, MA, USA). All newly determined sequences were deposited in Genbank (accession numbers KF250661–KF250761). Sequences were aligned and pairwise distances calculated in MEGA 5 (Tamura *et al.* 2011). Phylogenetic analysis was computed by Bayesian inference using MrBayes V.3.1.2. (Ronquist & Huelsenbeck 2003) after determining the appropriate model of nucleotide substitution under the Akaike information criterion implemented in MrModeltest (Nylander 2004; a general time-reversible model, GTR+I+G). We ran four Markov chains for 10 million generations, sampled every 1000 generations, with a random starting tree and default priors. *Trachylepis tavaratra* was used as the outgroup.

## Results and discussion

The resulting phylogenetic tree (Figs 1–2) reveals several major mitochondrial lineages and reciprocal paraphyly of the two focal species, in agreement with the results of Boumans *et al.* (2007). We emphasize that we here present this tree merely to identify clusters of genetically homogeneous mtDNA and discuss their distribution. We do not suggest interpreting mitochondrial non-monophyly of *T. gravenhorstii* and *T. elegans* as indicative of these taxa containing cryptic species, given that the multi-gene phylogeny of Lima *et al.* (2013) identified each of these two species as monophyletic, in a multi-gene analysis that included four nuclear genes. In fact, our results highlight the need for caution when relying on pure DNA barcode analysis, as the p-distances calculated between the main subclades of these species (ranging from 6.3–14.5%) are well above the species-level threshold established for scincid species (6.1%, Nagy *et al.* 2012), but it is unlikely that each represents a distinct species.

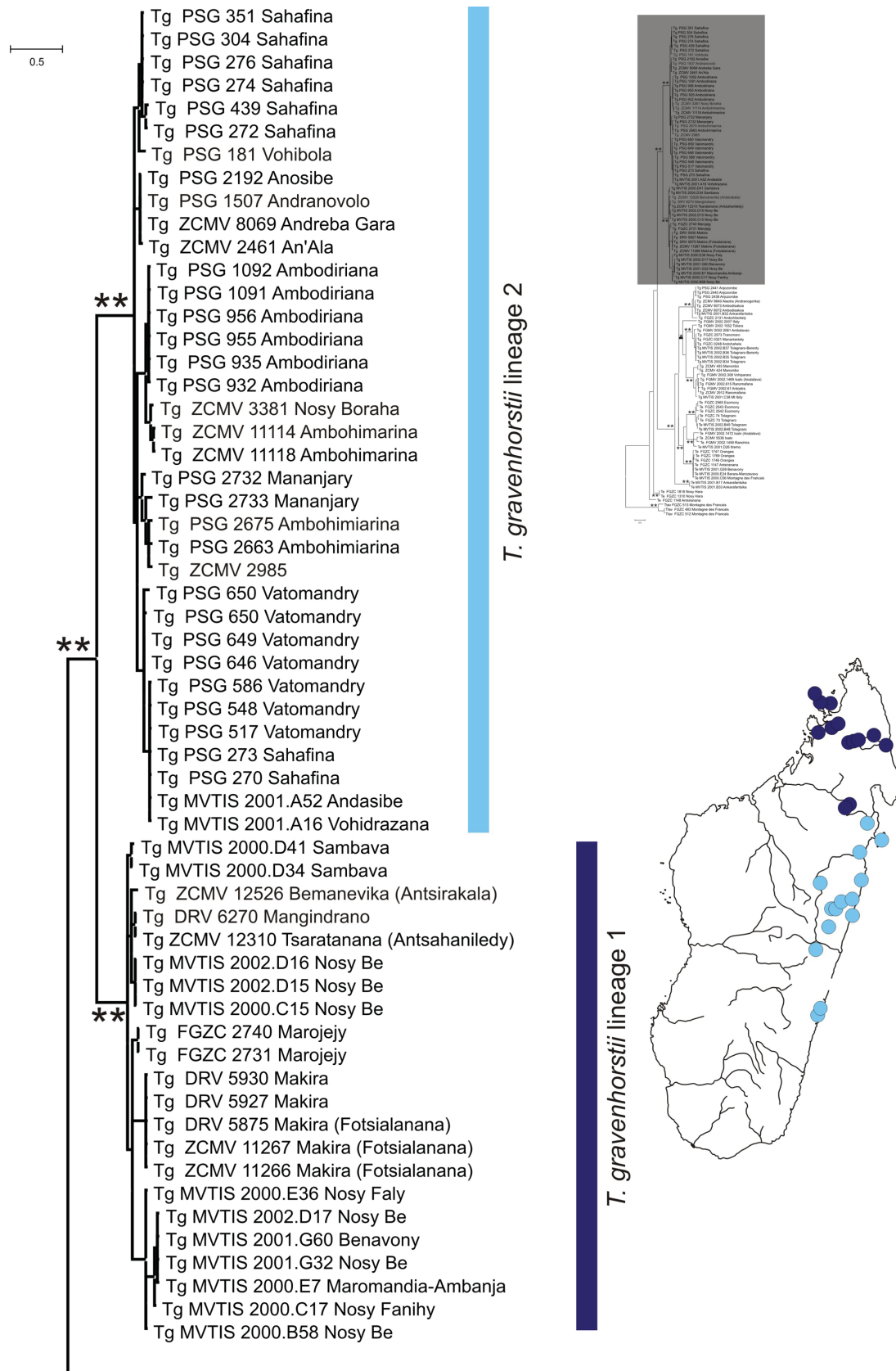
The first main clade in the analysis, supported by maximum posterior probability (PP) values of 1.0 (Fig. 1) contains two subclades (here symbolized by dark and light blue and named lineages 1 and 2). The entire clade corresponds to those populations marked in red color in the analysis of Boumans *et al.* (2007). They occur in the regions of the North East and Sambirano, reaching into the Northern Central East around the western edges of the Makira Massif (lineage 1), and in low to mid-elevations along the east coast, in the Northern Central East and Southern Central East Regions (lineage 2) (see also Fig. 3).

A second main clade in our analysis equally supported by maximum PP values contains the majority of *T. elegans* samples and four main clusters of *T. gravenhorstii* samples. These are here named as lineage 3, occurring in the North West and Northern Central East regions, and lineage 4a and 4b, occurring in the Southern Central East, South East, and South West regions (Fig. 2–3). Furthermore, a single sample from the South West (Ifaty) had a deviant sequence and can be seen as representing one further main mitochondrial lineage.

Samples of *T. elegans* from two northern localities (Nosy Hara and Antsiranana) are placed outside of the second main clade mentioned in the previous paragraph, although other samples from northern localities (Antsiranana, Orangea, Montagne des Français) are included therein. The two specimens collected near Antsiranana, representing lineages A and D of *T. elegans*, were even trapped in the same pitfall line. *Trachylepis elegans* and *T. gravenhorstii* are well-differentiated morphologically despite some overlap in most of the distinguishing characters (Brygoo 1983; Andreone & Greer 2002; Lima *et al.* in press), and one of the genetically deviant specimens (FGZC 1818 = ZSM 1549/2008 from Nosy Hara) which has been included in the morphological assessment, did not show any striking differences to the other *T. elegans* specimens which would indicate taxonomic distinctness (Lima *et al.* in press). One possible explanation for the mitochondrial paraphyly of *T. elegans* is a convoluted evolutionary history with mitochondrial introgression upon occasional hybridization with the sympatric *T. gravenhorstii*. Because *T. gravenhorstii* apparently does not occur nowadays in the extreme north of Madagascar, where the two genetically deviant *T. elegans* lineages were found (Nosy Hara, Antsiranana; Glaw & Vences 2007), this hypothesis of hybridization is however difficult to imagine at least for this region, given the current distribution patterns.

Within *T. gravenhorstii*, the COI data support more clearly than the 16S data of Boumans *et al.* (2007) the existence of two main entities: one containing lineages 1–2, and another one containing lineages 3–4 and the Ifaty population. Lima *et al.* (in press) found only faint morphological differences between specimens belonging to the main mtDNA clades (represented by lineages 1 and 4), but the number of specimens analysed was small. So far, no

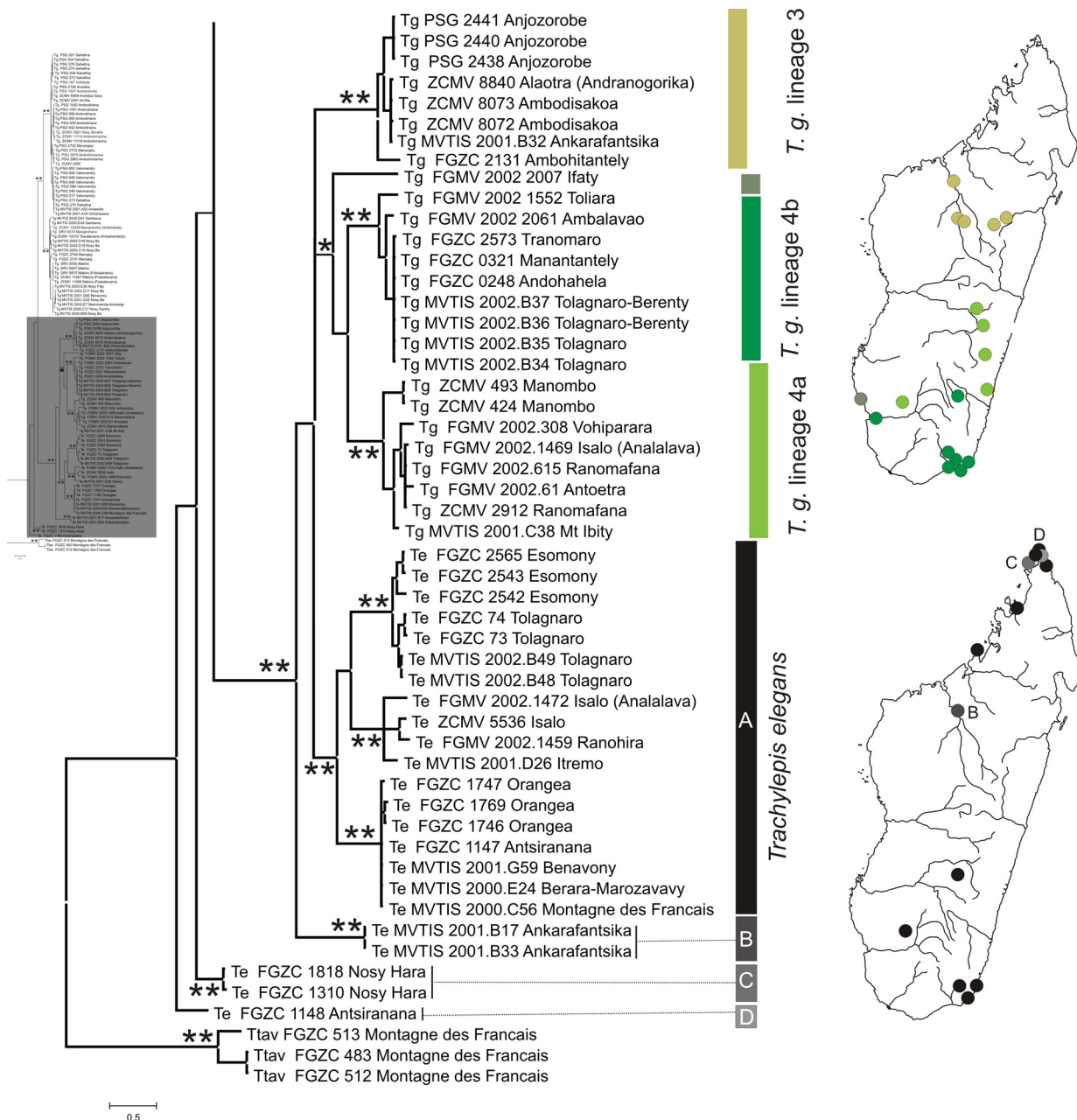
cases of sympatry have been observed, but our data allow localizing more precisely the contact zones. The two main mtDNA clades can be expected to be in contact or occur sympatrically in the area of the upper Mangoro river and Alaotra Lake where lineage 2 occurs, for instance in Andreba Gara at the south-eastern shore of the lake, while



**FIGURE 1.** Phylogenetic tree obtained by Bayesian Inference (50%-majority rule consensus tree) using 612 bp of the



mitochondrial COI gene of specimens of *Trachylepis elegans* and *T. gravenhorstii*. The inset picture schematically shows the entire tree, while the large figure shows its upper half in detail. The map indicates the provenance of the samples of lineages 1 and 2 (dark blue and light blue) of *T. gravenhorstii* (Tg). Asterisks mark Bayesian posterior probability support (two asterisks, PP=1.00) which is only shown for deeper nodes and not for within-lineage nodes. See Fig. 2 for the second part of this tree.



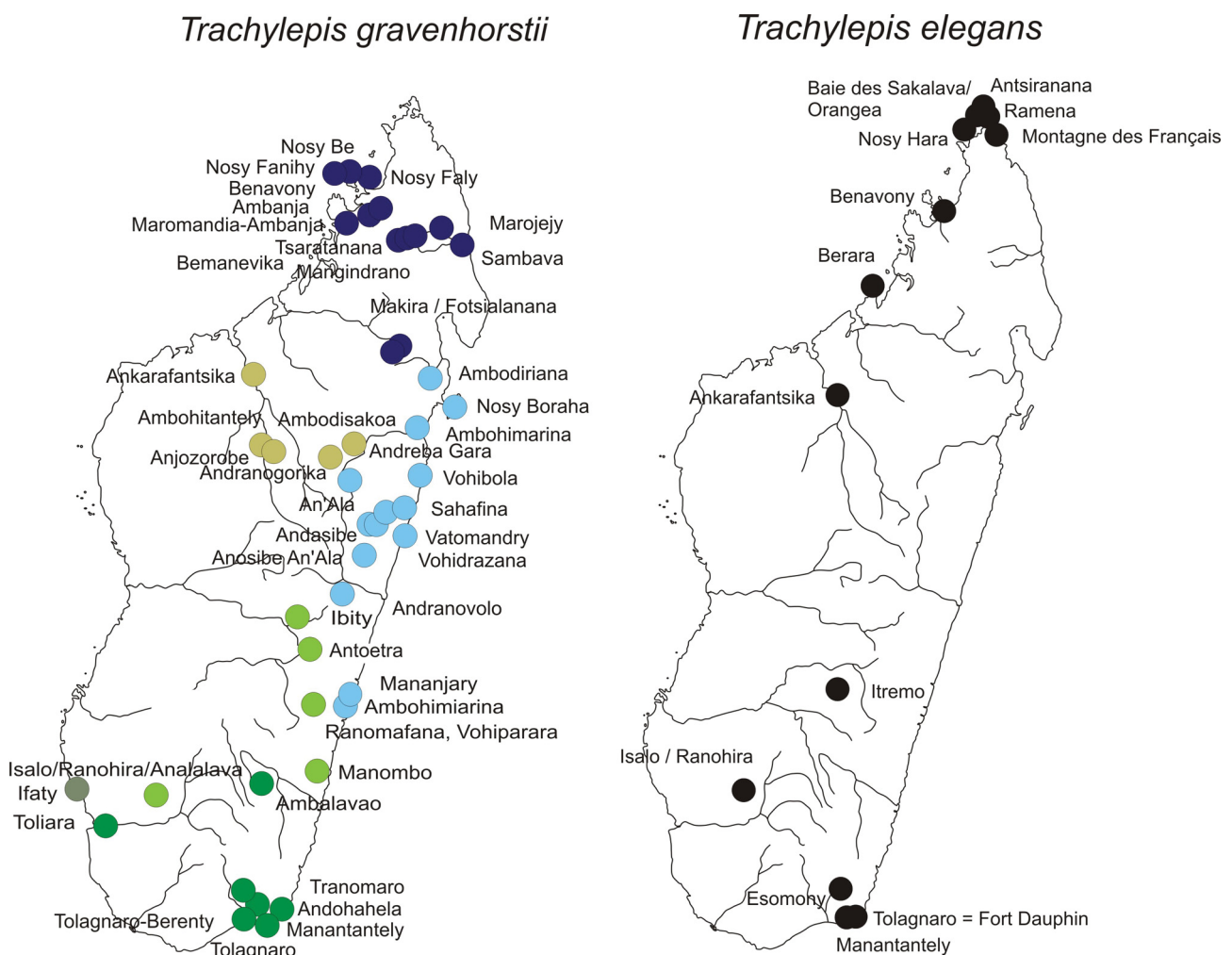
**FIGURE 2.** Phylogenetic tree obtained by Bayesian Inference (50%-majority rule consensus tree) using 612 bp of the mitochondrial COI gene of specimens of *Trachylepis elegans* and *T. gravenhorstii*. The inset picture schematically shows the entire tree, and the large figure shows its lower half in detail. The maps indicate the provenance of the samples of lineages 3 (green-yellow), 4a (light green), and 4b (dark green) of *T. gravenhorstii* (Tg), and of *T. elegans* (Te; clades in black (A) and shades of gray (B,C,D)). Asterisks mark Bayesian posterior probability support (two asterisks, PP=1.00; one asterisk, PP=0.95-0.99) which is only shown for deeper nodes and not for within-lineage nodes. Three sequences of *T. tavaratra* from Montagne des Français were used as the outgroup. See Fig. 1 for the first part of this tree.

lineage 3 is found in Ambodisakoa close to the northeastern shore of the lake and in Andranogorika not far from the west shore. A further contact zone probably occurs in the Southern Central East, where lineage 2 reaches along the lowlands to the Mananjary region whereas lineage 4a occurs at mid-altitudes around Ranomafana. A denser sampling of altitudinal and vegetational transects in this region might reveal cases of sympatric occurrence of these

lineages which then could be studied more in depth for differentiation and admixture (or absence thereof) in nuclear markers, and possible ecological specialization, in order to conclusively understand their status as either cryptic species or deep conspecific (mitochondrial) lineages.

The COI data reveal several phylogeographic patterns in *T. gravenhorstii* which agree with other widespread organisms in Madagascar. One of these patterns is the occurrence of lineage 3 at the western edge of the eastern rainforest band in the Upper Mangoro / Lake Alaotra region, spreading into the North West (around Ankarafantsika). This range agrees with a retreat-dispersion watershed defined by Wilmé *et al.* (2006) and suggests these lizards have been using the Betsiboka and Mangoro rivers as corridors, maintaining gene flow among western and eastern populations.

A second aspect refers to the low differences among haplotypes of lineage 2. In this lineage the populations occurring south of the Mangoro river (Mananjary, Ambohimiarina, and Andranovolo) have only weak differences to those occurring north of the river, suggesting that they might have expanded southwards only relatively recently. The fact that the Andranovolo haplotype does not cluster with the Mananjary/Ambohimiarina haplotypes might suggest two separate instances of cross-river migration. The Mangoro river therefore does not seem to constitute a barrier to gene flow and dispersal of these lizards, different from the situation in at least some amphibians (Gehring *et al.* 2012a). This agrees with data on Amazonian *Mabuya* (Miralles & Carranza 2010) where rivers were found to be dispersal corridors in some cases (*M. altamazonica* and *M. bistrata*), while they do not constitute strict geographical barriers for others (e.g., *M. nigropunctata* species complex).



**FIGURE 3.** Detailed maps with the sampled localities of *Trachylepis elegans* and of *T. gravenhorstii* (lineages colored as in Figs. 1–2).

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